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Molecular study of arbuscular mycorrhizal fungi colonizing the sporophyte of the eusporangiate rattlesnake fern (*Botrychium virginianum*, Ophioglossaceae)

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Abstract The arbuscular mycorrhizal (AM) fungi colonizing the sporophytes of the eusporangiate rattlesnake fern (*Botrychium virginianum*, Ophioglossaceae) in its Hungarian population were investigated in the present study. Different regions of the nrRNA gene complex were analyzed using two different primer sets. These produced similar results for the detected AM fungi phylotypes. Several AM fungal lineages were associated with sporophytes of *B. virginianum*. Phylogenetic analyses of different partial small subunit datasets grouped one lineage into the Gigasporaceae, showing similarities with *Scutellospora* sequences. In addition to unidentified *Scutellospora* phylotypes, it is possible that *S. gregaria* also colonized the fern. Several AM fungal phylotypes colonizing the sporophytes grouped into *Glomus* group A. They did not form distinct clades but grouped with sequences of AM fungi with different geographic and host origins. One main lineage clustered into the widespread *G. fasciculatum*/*G. intraradices* group and one into the subgroup GlGrAc, while others had no affinity to the subgroups of *Glomus* group A. As AM fungal phylotypes associated with *B. virginianum* seem to belong to widespread AM fungal taxa and show no specificity to this fern, we suppose that the previously described special anatomy of AM of *B. virginianum* is determined by the plant.

Keywords Arbuscular mycorrhiza · Fern · Molecular diversity · Polymerase chain reaction · Primers

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Introduction

The diversification of the monophyletic fungal phylum Glomeromycota might be synchronous with the spread of terrestrial plants (Simon et al. 1993). Almost all main terrestrial plant groups have arbuscular mycorrhiza (AM)-forming capacities, including members of either basal or derived plant groups (Wang and Qiu 2006). Several "lower" plants (i.e., cryptogamic, spore-dispersed plants) form AM, like, for example, basal lineages of liverworts (Kottke and Nebel 2005), *Marchantia* species (Russel and Bulman 2005), members of Lycopsida, and horsetails (Read et al. 2000; Brundrett 2002; Wang and Qiu 2006). The main groups of ferns differ in their mycorrhiza-forming features (Read et al. 2000; Zhao 2000; Brundrett 2002; Zhang et al. 2004; Turnau et al. 2005; Wang and Qiu 2006). The rattlesnake fern (*Botrychium virginianum* (L.) Sw.) is an eusporangiate fern, and most of the hitherto studied species of this group form AM. These ferns are associated with fungi in both parts of their life cycle. The achlorophyllous gametophytes of the Psilotales and Ophioglossales are obligate biotrophs and are associated with fungi with characteristics resembling Glomeromycota (Read et al. 2000; Brundrett 2002). The sporophytes of the Ophioglossaceae unambiguously form AM. This family represents an early branched eusporangiate fern group (Pryer et al. 2001) with three genera (Wagner 1990) and includes many

protected species. Gallaud 1905 documented the AM of *Ophioglossum* and grouped it into the *Arum*-type. The anatomical characteristics of the fungal associations of the sporophytes from this group have since been described in several studies (see in Kovács et al. (2003) and references therein). Specific arbuscule structures have been reported from *Ophioglossum* species, such as the "Sternarbuskel" with swollen branch ends (Burgeff 1938; Schmid and Oberwinkler 1996) or coralloid arbuscules (Schmid and Oberwinkler 1996), while arbuscules with lobe-like branches, resembling the structures found in Triassic fossils (Stubblefield et al. 1987), have been described from the sporophyte of *B. virginianum* (Kovács et al. 2003).

Despite the numerous anatomical studies of AM of ferns, we are not aware of any published, detailed molecular investigation of AM fungi colonizing ferns except for a study on *Botrychium pumicola* Coville (Camacho and Trappe 1998).

Molecular phylogenetic studies of the Glomeromycota have revealed considerable diversity in this phylum (Öpik et al. 2006). The relatively few species described mostly based on the spore morphologies probably represent only a small part of the existing taxa. Although partial large subunit (LSU) sequences of nrDNA (e.g., van Tuinen et al. 1998; Geue and Hock 2004; da Silva et al. 2006; Gamper and Leuchtmann 2007) and actin and elongation factor 1-alpha sequences have been used to study the Glomeromycota (Helgason et al. 2003), sequences of the small subunit (SSU) gene and internal transcribed spacer (ITS) region of the nrDNA have most frequently been used for in planta molecular studies of AM fungi (e.g., Husband et al. 2002; Wubet et al. 2003; Douhan et al. 2005; Rodríguez-Echeverría and Freitas 2006; Wubet et al. 2006a, b). The number of molecular in planta diversity studies of AM fungi has been increasing rapidly during the past few years. However, there is an uneven distribution of data concerning geographic origins or host plants (Öpik et al. 2006), similar to those based on spore studies (Morton 1993). There are very few AM fungal data from the Carpathian Basin in public molecular databases. Until the beginning of the present work here, only three AM fungal sequences from Hungary had been released, two from spores of *Glomus* species (Landwehr et al. 2002) and one originating from a tree nursery experiment (Szegő et al. 2007).

The unique Hungarian population of the strictly protected, red-listed rattlesnake fern is one of the biggest populations of this species in Europe with approximately 1,000 adult sporophytes (Csiky 1997; Bagi 2006). Our earlier ultrastructural study (Kovács et al. 2003) indicated that the fungal colonization of *B. virginianum* sporophytes in the area could be considered as a functional AM and not just an occasional colonization by Glomeromycota.

The main aim of the work presented here was to carry out molecular characterization of the AM fungi colonizing the sporophyte of *B. virginianum*, using different Glomeromycota-specific polymerase chain reaction (PCR) primer combinations targeting the nrDNA gene complex. Primers designed to target the SSU 5′ part and the ITS region of nrDNA (Redecker 2000; Redecker et al. 2003) and another recently published primer set amplifying a part of the SSU (Wubet et al. 2006a) were used in our study. Comparison of results obtained with these two PCR systems is discussed.

Materials and methods

Sampling area

The unique Hungarian population of *B. virginianum* occurs on the Great Hungarian Plain. The plant community of the habitat is a mixed deciduous woodland dominated by AM-forming species (Csiky 1997; Kovács and Bagi 2001). The mean annual precipitation in the area is 550–600 mm, and the mean annual temperature is 10.7°C. The thickness of the weakly calcareous (pH 7.5–8) sandy upper layer of the soil is varied between 0.5 and 1 m and contains 3.3–8.5% organic matter. The area is protected under the supervision of the Kiskunság National Park.

Sample collecting

The officially permitted collecting of root samples from five mature *B. virginianum* sporophytes was carried out between October 2004 and October 2005. The collected roots were checked under a stereomicroscope, and the external debris was removed. To examine fungal colonization, thin cross-sections were cut with a razor blade every 5–7 mm and stained in aniline blue. If hyphal colonization resembling those formed by AM fungi was detected, that root segment was used in the subsequent molecular work.

DNA extraction, amplification, cloning and sequencing

Total DNA was extracted from the root samples using a DNAeasy Mini Kit (Qiagen, Hilden, Germany). In the first step, the roots were homogenized in Eppendorf tubes in the extraction buffer (AP1) using micropestles and sand. Afterwards, the instructions of the manufacturer were followed. Seminested PCR were carried out with the primers described by (1) Redecker (2000) and Redecker et al. (2003) and (2) Wubet et al. (2006a) as follows. (1) Primers NS5 and ITS4 (White et al. 1990) were used in the first step with the following PCR program: denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 35 s, extension at 72°C for 1 min and 50 s, and final

extension at 72°C for 10 min. The reaction mixtures with or without detectable amplicons were diluted 1,000 and 100 times, respectively, and used as a target in the second reaction. Amplification was always checked in ethidium bromide-stained 1% agarose gels. In the second step, the primer pairs used were GIGA1313-ITS4 and GLOM5.8R-NS5 with 50°C, ACAU1661-ITS4, ARCH1311-ITS4, and GLOM1310-ITS4 with 52°C, LETC1670-ITS4, LETC1677-ITS4, and GIGA5.8R-NS5 with 54°C as the annealing temperatures. The rest of the PCR conditions were identical to those described above. (2) In the first step of the nested PCR targeting the partial SSU of nrDNA (Wubet et al. 2006a), the GLOM1536 and GlomerTW0 primers were used with the following PCR conditions: denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 35 s, extension at 72°C for 1 min and 40 s, and final extension at 72°C for 10 min. One microliter of the first reaction was used as a target in the second step using the primers GlomerTW1, GlomerTW2, GlomerTW3, and GlomerTW4; all of them were paired with GlomerTW0. The PCR conditions were identical in the first and the second step of the seminested PCRs. All the reaction mixtures contained 0.1 volume 10× PCR buffer (MBI Fermentas, Vilnius, Lithuania), 200 μM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas), 0.5 μM of each primer, 1 U recombinant *Taq* DNA polymerase (MBI Fermentas). All amplifications were carried out using a T-Gradient 96 thermocycler (Biometra, Göttingen, Germany) with ramp speed set to 1°C/s.

When appropriate-sized PCR products were detected in agarose gels, these were cleaned using a PCR Clean up-M kit (Viogene, Hong Kong, China). When amplicons with different sizes were observed, the appropriate sized bands were isolated from agarose gels using a Gel-M Extraction System (Viogene). PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI), and the clones were transformed into competent JM109 *Escherichia coli* cells (Promega) by heat shock. After overnight growing of white colonies picked into 2-ml Luria–Bertani medium, the plasmid DNA was cleaned using a Mini-M Plasmid DNA Extraction Kit (Viogene). The clones containing an appropriate-sized insert were selected after digestion with *Eco*RI restriction enzyme (MBI Fermentas). Positive clones were sequenced on both strands with universal forward and reverse primers using an ABI PRISM 3.1 BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA). Electrophoresis was carried out in an ABI PRISM 3100 Genetic Analyzer at the service laboratory of the Biology Research Center (Szeged, Hungary). DNA sequences were processed and analyzed with the Staden Program Package (Staden et al. 2000). Non-Glomeromycota sequences were excluded from the analyses after the nucleotide basic local alignment search tool (BLAST; Altschul et al. 1990) homology search on National Center for Biotechnology Information (http://www.ncbi.nih.gov/BLAST). To exclude chimeric sequences from further analyses, the Chimera Check program of the Ribosomal Database Project II (Cole et al. 2003) was used (http://rdp8.cme.msu.edu/html/). Altogether, 110 clones were sequenced, approximately 70% of which were usable Glomeromycota sequences. All the sequences used in the analyses presented here have been deposited into the GenBank under accession numbers EF393579–EF393618.

Phylogenetic analyses

The two seminested PCR sets amplified different regions of nrDNA that overlapped only on a short region of the SSU gene. As a consequence, the sequences obtained with the two systems were analyzed separately. These were marked as "RED" and "WUB" for Redecker's and Wubet's primer sets, respectively. Two data matrices were constructed from both RED and WUB sequences. To study the position of the detected AM fungi lineages within the phylum Glomeromycota, representatives of both RED and WUB sequences were aligned with SSU ribosomal DNA sequences from Schüßler et al. (2001), complemented with sequences from other works (Walker et al. 2004; de Souza et al. 2005). These were marked as RED-PHYL and WUB-PHYL, respectively. The sequence of *Endogone pisiformis* (X58724) was used as the outgroup during these analyses.

Most of the phylotypes detected in *B. virginianum* belonged to *Glomus* group A (GlGrA). The representatives of this group from both RED and WUB datasets, together with representatives of similar sequences found during the BLAST search, were analyzed together with the GlGrA AM fungi sequences from *B. viginianum*. These datasets were marked as RED-GlgrA and WUB-GlgrA, highlighting that these analyses were restricted to GlGrA. The sequence of *Glomus lamellosum* (AJ276087) was used as the outgroup during these analyses.

The sequences were aligned by MultAlin (Corpet 1988) from its website (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and checked and edited manually with the program ProSeq2.9 (Filatov 2002). Several putative analyses with different versions of the databases, including different alignments, were carried out using the Kimura two-parameter model and neighbor-joining algorithm with the program MEGA 3.1 (Kumar et al. 2004). The maximum-likelihood (ML) phylogenetic analyses were performed using the program PHYML (Guindon and Gascuel 2003). According to Modeltest 3.06 (Posada and Crandall 1998), the general time reversible nucleotide substitution model was used with ML estimation of base frequencies. The proportion of the invariable sites was estimated and optimized. Four substitution rate categories

were set, and the gamma distribution parameter was estimated and optimized. Bootstrap analysis with 1,000 replicates was used to test the statistical support of the branches. The same substitution model was used in the Bayesian analyses performed with the program MrBayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The Markov chain was run over 2,000,000 generations, sampling in every 100 step and with a burn in at 7,500 sampled trees. The details of the analyses and the alignments are available upon request. The phylogenetic trees were visualized and edited by the Tree Explorer of the MEGA 3.1 program (Kumar et al. 2004).

Results

Root colonization

The characteristics of fungal colonization of the sporophyte of *B. virginianum* were similar to those reported in previous anatomical studies carried out on the fern (Kovács and Bagi 2001, Kovács et al. 2003). Approximately one third of the roots examined in this work were colonized by hyphae localized in a two- to four-cell layer of the root cortex (Fig. 1).

Specificity of the primers

Nonspecific amplifications were repeatedly obtained with both PCR systems. Two kinds of nonspecificity could be distinguished: amplification of Glomeromycota nrDNA from groups different from the targeted taxa and the amplification of non-Glomeromycota nrDNA. Both kinds of nonspecificity were detected using the primers from Redecker (2000) and Redecker et al. (2003), while only non-Glomeromycota DNA amplifications were detected using the primers designed by Wubet et al. (2006a, b). The portion of the non-Glomeromycota sequences was generally low (approx. 15%). However, there were considerable differences between the primers targeting different groups; the oligonucleotides designed to amplify basal branches of the Glomeromycota amplified more non-Glomeromycota nrDNA than the primers of other Glomeromycota. All of the non-Glomeromycota fungal sequences were Ascomycota sequences.

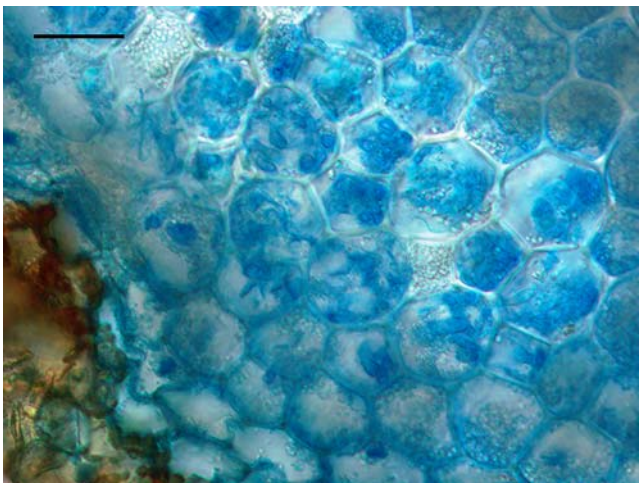

Fig. 1 Cross section of the root of the sporophyte of *Botrychium virginianum* illustrating the fungal colonization of cortical cells. *Bar*: 40 μm

Phylogenetic analyses

All the methods used for inferring phylogenies from the different datasets resulted in very similar results. The analyses of two datasets are presented below in detail: (1) the results obtained using the RED-PHYL dataset to present the position of the newly detected phylotypes within the Glomeromycota (Fig. 2) and (2) the results obtained from the WUB-GlgrA dataset to show the position of the AM fungi of *B. virgianum* within the GlGrA together with representatives of sequences obtained with BLAST (Fig. 3).

The position of AM fungal phylotypes colonizing *B. virginianum*

The RED-PHYL dataset contained 76 sequences and was 432 characters long. The inferred phylogeny of the phylum was in accordance with the known grouping of the Glomeromycota (Fig. 2). The phylotypes of AM fungi colonizing the sporophyte of *B. virginianum* separated into five main groups, one grouped into the Gigasporaceae and four into Glomeraceae, exclusively into the GlGrA (Fig. 2). The analyses of the WUB-PHYL dataset resulted similar branching of the phylotypes (data not shown). *Scutellospora* sequences (BV-RED-1) detected in sporophytes of *B. virginianum* formed two lineages: one showed similarities with sequences of *S. castanea* C. Walker/*S. fulgida* Koske & C. Walker, while the other showed unambiguous similarity with the sequences of *S. gregaria* (N.C. Schenck & T.H. Nicolson) C. Walker & F.E. Sanders (Fig. 2).

Four main groups were formed within the GlGrA (Fig. 2). Two of them clustered unambiguously into the previously separated subgroups of GlGrA (according to Schwarzott et al. 2001): One (BV-RED-3) grouped into the subgroup GlGrAb, forming a distinct clade with moderate support (Fig. 2), while the other (BV-RED-2) formed a well-supported clade with *Glomus* sp. W3347 representing the subgroup GlGrAc (Fig. 2). The remaining two groups, BV-RED-4 and BV-RED-5, formed distinct lineages with moderate support in the case of BV-RED-4 and with ambiguous branching for BV-RED-5 (Fig. 2). No sequence obtained during the BLAST analyses grouped unambigu-

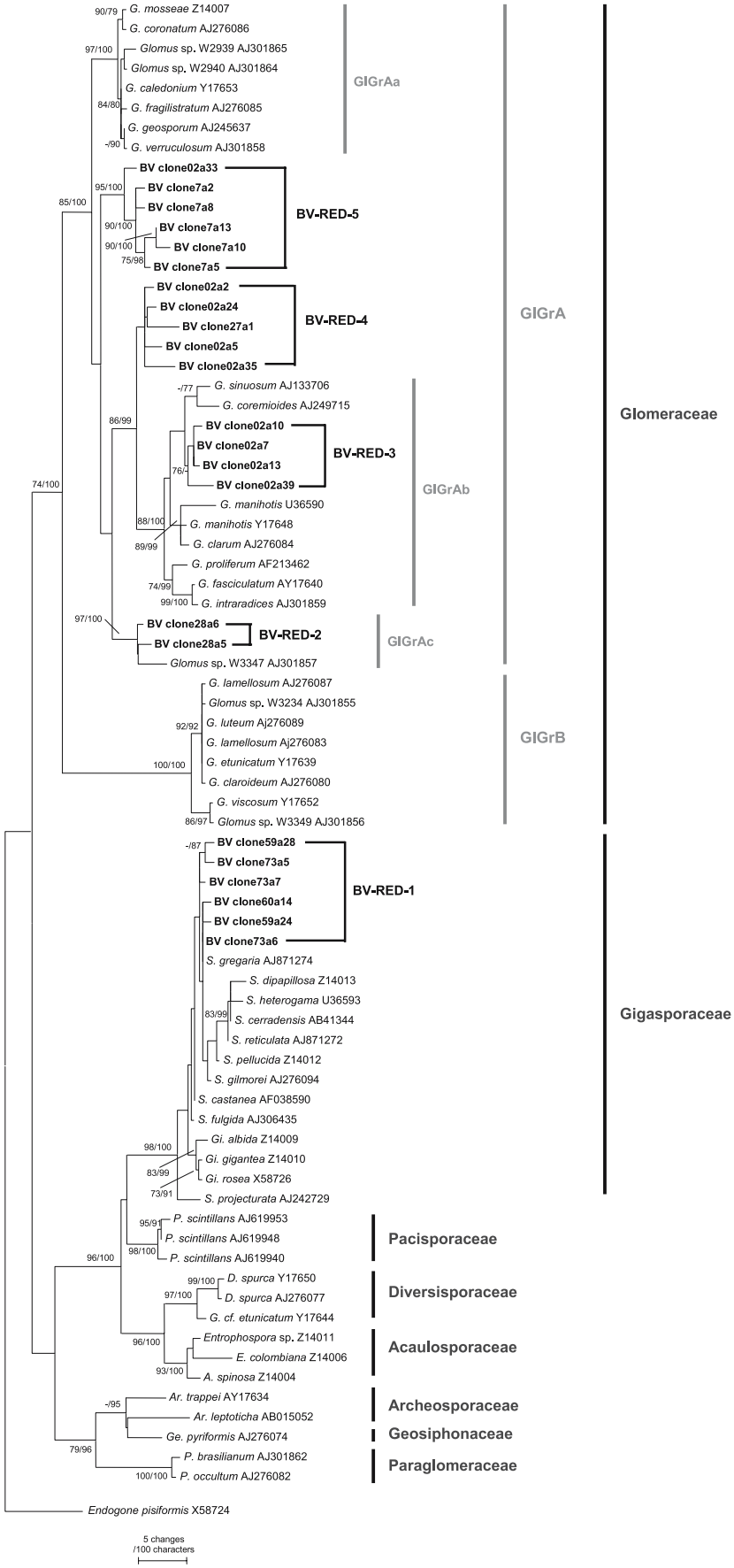

Fig. 2 The maximum-likelihood tree inferred from partial SSU sequences showing the position of the AM fungi colonizing *B. virginianum* in the phylum Glomeromycota (*BV-RED-1–5*). The sequences obtained in this study with the primers designed by Redecker (2000) and Redecker et al. (2003) are in *bold*. *GlGrAa*, *GlGrAb*, and *GlGrAc* correspond to the subgroups of GlGrA following Schwarzott et al. (2001). The first value at the braches shows the bootstrap values as percentage, while the values after the slash are the posterior probabilities as percentages. The values below 70% are not shown. Genera: *G. Glomus*, *Gi. Gigaspora*, *S. Scutellospora*, *P. Pacispora*, *D. Diversispora*, *E. Enthrophospora*, *A. Acaulospora*, *Ar. Archeospora*, *Ge. Geosiphon*, *P. Paraglomus*. The GenBank accession numbers are indicated after taxon names

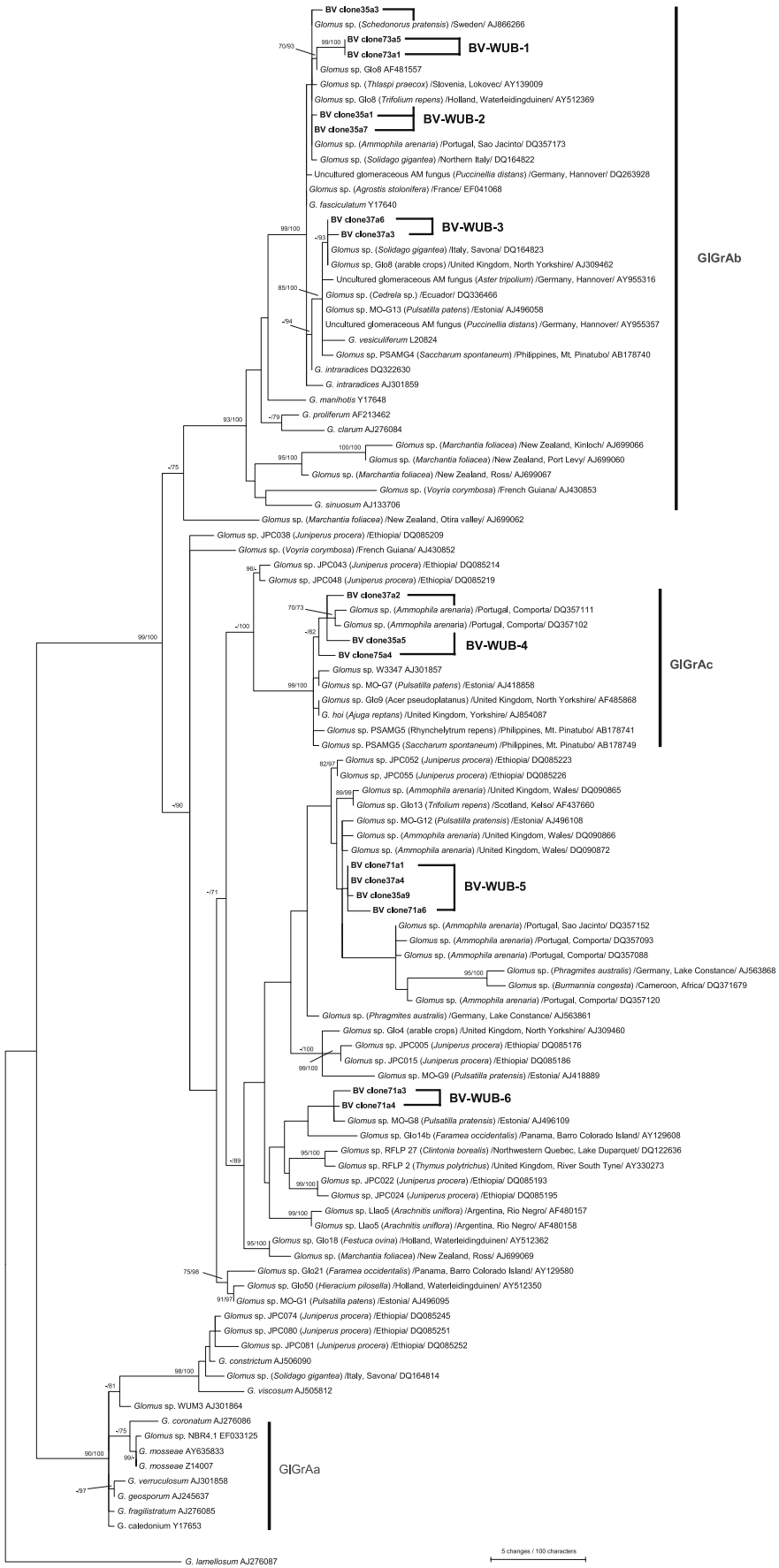

Fig. 3 The maximum-likelihood tree inferred from partial SSU sequences showing the position of the AM fungi colonizing *B. virginianum* within *Glomus* group A (*BV-WUB-1–6*). The sequences obtained in this study with the primers designed by Wubet et al. (2006a) are in *bold*. *GlGrAa*, *GlGrAb*, and *GlGrAc* correspond to the subgroups of GlGrA following Schwarzott et al. (2001). The first value at the branches shows the bootstrap values as percentage, while the values after the slash are the posterior probabilities as percentages. The values below 70% are not shown. The accession number of each GenBank data has been indicated. The hosts (when it was known) are presented in *parenthesis*, while the geographic origin is given between *slashes*

ously with these newly obtained AM fungal sequences from *B. virginianum*.

The AM fungal phylotypes colonizing *B. virginianum* within the *Glomus* Group A

Numerous similar AM fungal sequences obtained from different geographic regions and hosts were collected in the BLAST analysis of the GlGrA sequences obtained with the WUB primers. The WUB-GlgrA dataset contained 103 sequences and was 464 characters long. Six groups of AM fungal sequences from *B. virginianum* were separated, generally with moderate support (Fig. 3). The sequences within the subgroup GlGrAb formed three lineages rather than forming one group (Fig. 3). Only the group BV-WUB-1 separated unambiguously from any known sequences, while the BV-WUB-2 and BV-WUB-3 grouped together with sequences of known taxa and also with sequences of uncultured *Glomus* spp. originating from in planta studies of different hosts carried out in different geographic regions including even different continents (Fig. 3). One lineage (BV-WUB-4) grouped into the well-supported subgroup GlGrAc and branched together with AM fungal sequences from different regions and host plants (Fig. 3). A high number of sequences belonging to GlGrA formed a large group without affinity to the subgroups of GlGrA (Fig. 3). No described taxon could be found in this group. Two groups of sequences of AM fungal colonizing *B. virginianum* (BV-WUB-5 and BV-WUB-6) branched within this group showing similarities with AM fungal sequences from different hosts and geographic regions (Fig. 3).

Discussion

The previous anatomical study of the sporophyte of *B. virginianum* unambiguously showed that the species form AM, similarly to other ferns of the family Ophioglossaceae (Kovács et al. 2003). The present in planta molecular study of AM fungi colonizing *B. virginianum* revealed that several different Glomeromycota colonize the sporophyte of the eusporangiate fern. Both PCR sets in our study have advantages, and in spite of the limitations of the comparability of the results obtained, they produced similar results. In certain cases, the use of the more specific 'Redecker' primers (Redecker 2000; Redecker et al. 2003) could be more advantageous than those published by Wubet et al. (2006a). The GIGA5.8R-NS5 and GIGA1313-ITS4 primer pairs were designed to amplify only the Gigasporaceae (Redecker 2000; Redecker et al. 2003), while the primers GlomerTW0–GlomerTW4 were designed to amplify Gigasporaceae and also Glomeraceae, Pacisporaceae, and Paraglomeraceae (Wubet et al. 2006a). In addition to more frequent non-specific amplification by the GlomerTW0–GlomerTW4 primers, several *Glomus* sequences were obtained with these primers. If the root colonization is dominated by *Glomus* taxa, the detection of the Gigasporaceae and other sequences will be more difficult with the GlomerTW0–GlomerTW4 primers. On the other hand, as nearly complete SSU gene sequences are generally used in phylogenetic and taxonomical studies of the phylum (Schüßler et al. 2001; Schwarzott et al. 2001; Walker et al. 2004; de Souza et al. 2005), the whole length (approx. 1,000 bp) of the sequences obtained using the GlomwerTW primers could be used to analyze the position of the phylotypes within the Glomeromycota. Numerous in planta molecular studies of AM fungal diversity (e.g., Helgason et al. 2002; Husband et al. 2002; Öpik et al. 2003; Whitfield et al. 2004; Douhan et al. 2005; Vallino et al. 2006; Wolfe et al. 2007) used primers NS31 (Simon et al. 1992) and AM1 (Helgason et al. 1998). As the SSU segment amplified by this primer pair overlaps with a part of the nrDNA amplified by GlomerTW primers, numerous sequences could be analyzed together with the newly obtained ones. This could prevent misleading interpretation of distinct lineages when the separation of the groups is not due to a real segregation but only to lack of data. In the present study, the distinct groups obtained in the analyses of the datasets from "Redecker" primers raised the question whether the separation is due to specificity (regardless of being geographic, habitat, or host specificity). However, the lineages were not distinct anymore when, because of overlap, other sequences from the GenBank could be included into the analyses of the data from GlomerTW primers, and the sequences grouped together with the phylotypes of the AM fungi colonizing *B. virginianum*.

A rough estimation of the average number of AM fungal lineages of main habitat types, which could be considered as a kind of diversity, was calculated by Öpik et al. (2006) based on a meta-analysis of the results from molecular studies of AM fungal diversity. The habitat of the *B. virginianum* is a forest on sandy soil in a continental climate, and the number of AM fungal lineages found (five or six to seven, depending on the dataset) is in accordance with the range calculated by Öpik et al. (2006) for these habitats.

None of the phylotypes of AM fungi colonizing *B. virginianum* were distinct. All the lineages branched in the GlGrA and clustered with common taxa from different geographic and host origin. AM fungi of the *G. intraradices*/*fasciculatum* group, which is one of the most common Glomeromycota groups, also colonized the sporophyte of *B. virginianum*. Among the AM fungal lineages detected in *B. virginianum*, several other sequences grouped with *Glomus* sp. W3347 representing the subgroup GlGrAc (Schwarzott et al. 2001). The taxa grouping with *G. hoi* (Helgason et al. 2002) are also quite frequent and are common root-colonizing fungi; they have been detected in

several different environments, generally in natural, undisturbed habitats (Öpik et al. 2006). Many sequences, together with two lineages colonizing *B. virginianum*, did not cluster with the three subgroups of GlGrA but branched in a diverse group without strong statistical support as a whole. This diverse group seems to overlap with the "grassland" group sensu Öpik et al. (2006). The *Scutellospora* sequences detected in sporophytes of *B. virginianum* belonged to two lineages: One of them showed similarities with *S. castanea*/*S. fulgida* and separated from the widespread *Scutellospora* group (Öpik et al. 2006), while the other showed unambiguous similarity to *S. gregaria* (Nicolson and Schenck 1979; de Souza et al. 2005). To our knowledge, this is the first report of in planta detection of this taxon in a natural environment.

The molecular study of Glomeromycota colonizing the sporophyte of *B. virginianum* revealed that the fern is colonized by several different AM fungal lineages. These phylotypes are not distinct and do not show specificity; they group with widely distributed taxa and with AM fungal sequences of different geographic and host origin. Based on these results, we suppose that the previously described odd structure of AM of the *B. virginianum* sporophyte (Kovács et al. 2003) is determined by the fern.

The results presented here are among the first molecular data of AM fungi in the Carpathian Basin. Detailed information about AM fungi in a certain geographic area could help to control and plan application of AM fungal inoculation in a region.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bagi I (2006) Population structure and dynamics of rattlesnake fern (*Botrychium virginianum* (L.) Sw.) in Kunfehértó. Conference on the Actual Flora and Vegetation in the Carpathian Basin VII. Debrecen, Abstracts, p 6

Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. New Phytol 154:275–304

Burgeff H (1938) Mycorrhiza. In: Verdoorn F (ed) Manual of pteridology. Nijhoff, The Hague, pp 159–196

Camacho FJ, Trappe JM (1998) DNA examination of the root fungal community of pumice grape fern, *Botrychium pumicola*. 2nd ICOM, Abstracts, Uppsala, p 38

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31:442–443

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881–10890

Csiky J (1997) A *Botrychium virginianum* (L.) Sw. Fitocönológiai és ökológiai vizsgálata a kunfehértói holdrutás erdőben. Kitaibelia 2:56–68

Douhan GW, Petersen C, Bledsoe CS, Rizzo DM (2005) Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific application. Mycorrhiza 15:365–372

Filatov DA (2002) ProSeq: a software for preparation and evolutionary analysis of DNA sequence data sets. Mol Ecol Notes 2:621–624

Gallaud I (1905) Études sur les mycorrhizes endotrophes. Rev Gen Bot 17:5–48,66–83,123–136,223–239,313–325,425–433,479–500

Gamper H, Leuchtmann A (2007) Taxon-specific PCR primers to detect two inconspicuous arbuscular mycorrhizal fungi from temperate agricultural grassland. Mycorrhiza 17:145–152

Geue H, Hock B (2004) Determination of *Acaulospora longula* and *Glomus* subgroup Aa in plant roots from grassland using new primers against the large subunit ribosomal DNA. Mycol Res 108:76–83

Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431

Helgason T, Merryweather JW, Denison J, Wilson P, Young JPW, Fitter AH (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-occuring fungi and plants from temperate deciduous woodland. J Ecol 90:371–384

Helgason T, Watson IJ, Young PW (2003) Phylogeny of the Glomerales and Diversisporales (Fungi: Glomeromycota) from actin and elongation factor 1-alpha sequences. FEMS Microbiol Lett 229:127–132

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754–755

Husband R, Herre EA, Turner SL, Gallery R, Young PW (2002) Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in tropical forest. Mol Ecol 11:2669–2678

Kottke I, Nebel M (2005) The evolution of mycorrhiza-like associations in liverworts: an update. New Phytol 167:330–334

Kovács GM, Bagi I (2001) Mycorrhizal status of a mixed deciduous forest from the Great Hungarian Plain with special emphasis on the potential mycorrhizal partners of *Terfezia terfezioides* (Matt.) Trappe. Phyton 41:161–168

Kovács GM, Kottke I, Oberwinkler F (2003) Light and electron microscopic study on the mycorrhizae of sporophytes of *Botrychium virginianum*—arbuscular structures resembling fossil forms. Plant Biol 5:574–580

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163

Landwehr M, Hildebrandt U, Wilde P, Nawrath K, Tóth T, Biró B, Bothe H (2002) The arbuscular mycorrhizal fungus *Glomus geosporum* in European saline sodic and gypsum soils. Mycorrhiza 12:199–211

Morton JB (1993) Problems and solutions for the integration of glomalean taxonomy, systematic biology, and the study of endomycorrhizal phenomena. Mycorrhiza 2:97–109

Nicolson TH, Schenck NC (1979) Endogonaceous mycorrhizal endophytes in Florida. Mycologia 71:178–198

Öpik M, Moora M, Liira J, Kõljalg U, Zobel M, Sen R (2003) Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forests and grassland soils. New Phytol 160:581–593

Öpik M, Moora M, Liira J, Zobel M (2006) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J Ecol 94:778–790

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Pryer KM, Schneider H, Smith AR, Cranfill R, Wolf PG, Hunt JS, Sipes SD (2001) Horsetails and ferns are a monophyletic group and the closest relatives to seed plants. Nature 409:618–622

Read DJ, Ducket JG, Francis R, Ligrone R, Russel A (2000) Symbiotic fungal associations in "lower" plants. Philos Trans R Soc London B 355:815–831

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80

Redecker D, Hijri I, Wiemken A (2003) Molecular indetification of arbuscular mycorrhizal fungi in roots: perspectives and problems. Folia Geobot 38:113–124

Rodríguez-Echeverría S, Freitas H (2006) Diversity of AMF associated with *Ammophila arenaria* ssp. *arundinacea* in Portuguese sand dunes. Mycorrhiza 16:543–552

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Russel J, Bulman S (2005) The liverwort *Marchantia foliacea* forms specialized symbiosis with arbuscular mycorrhizal fungi in the genus *Glomus*. New Phytol 165:567–579

Schmid E, Oberwinkler F (1996) Light and electron microscopy of a distinctive VA mycorrhiza in mature sporophytes of *Ophioglossum reticulatum*. Mycol Res 100:843–849

Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421

Schwarzott D, Walker C, Schüßler A (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. Mol Phylogenet Evol 21:190–197

da Silva GA, Lumini E, Maia LC, Bonfante P, Bianciotto V (2006) Phylogenetic analysis of Glomeromycota by partial LSU rDNA sequences. Mycorrhiza 16:183–189

Simon L, Lalonde M, Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–293

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

de Souza FA, Declerck S, Smit E, Kowalchuk GA (2005) Morphological, ontogenetic and molecular characterization of *Scutellospora reticulata* (Glomeromycota). Mycol Res 109:697–706

Staden R, Beal KF, Bonfield JK (2000) The Staden package, 1998. Methods Mol Biol 132:115–130

Stubblefield SP, Taylor TN, Trappe JM (1987) Fossil mycorrhizae: a case for symbiosis. Science 237:59–60

Szegő D, Rudnóy S, Zöld-Balogh Á, Bratek Z (2007) Homoki szarvasgombával beoltott akáccsemeték mikorrhizáinak és gyökérasszociált gombáinak morfológiai és molekuláris vizsgálata. Mikol Közl (in press)

Turnau K, Anielska T, Jurkiewicz A (2005) Mycothallic/mycorrhizal symbiosis of chlorophyllous gametophytes and sporophytes of a fern, *Pellaea viridis* (Forsk.) Prantl (Pellaeaceae, Pteridales). Mycorrhiza 15:121–128

Vallino M, Massa N, Lumini E, Bianciotto V, Berta G, Bonfante P (2006) Assessment of arbuscular mycorrhizal fungal diversity in roots of *Solidago gigantea* growing in a polluted soil in Northern Italy. Environ Microbiol 8:971–983

van Tuinen D, Jacquot E, Zhao B, Gollotte A, Gianinazzi-Pearson V (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887

Wagner WH (1990) Ophioglossaceae. In: Kramer KU, Green PS (eds) The families and genera of vascular plants. Volume I. Pteridophytes and Gymnosperms. Springer, Berlin 193–197

Walker C, Blaszkowski J, Schüßler A (2004) *Gerdemannia* gen. nov., a genus separated from *Glomus*, and Gerdemanniaceae fam. nov., a new family in the Glomeromycota. Mycol Res 108:707–718

Wang B, Qiu Y-L (2006) Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16:299–363

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Brinsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322

Whitfield L, Richards AJ, Rimmer DL (2004) Relationships between soil heavy metal concentration and mycorrhizal colonisation in *Thymus polytrichus* in northern England. Mycorrhiza 14:55–62

Wolfe BE, Mummey DL, Rillig MC, Klironomos JN (2007) Small-scale spatial heterogeneity of arbuscular mycorrhizal fungal abundance and community composition in a wetland plant community. Mycorrhiza 17:175–183

Wubet T, Weiss M, Kottke I, Teketay D, Oberwinkler F (2003) Molecular diversity of arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia. New Phytol 161:517–528

Wubet T, Weiss M, Kottke I, Teketay D, Oberwinkler F (2006a) Phylogenetic analysis of nuclear small subunit rDNA sequences suggests that the endangered African Pencil Cedar, *Juniperus procera*, is associated with distinct members of Glomeraceae. Mycol Res 110:1059–1069

Wubet T, Weiss M, Kottke I, Oberwinkler F (2006b) Two threatened coexisting indigenous conifer species in the dry Afromontane forests of Ethiopia are associated with distinct arbuscular mycorrhizal communities. Cab J Bot 84:1617–1627

Zhang Y, Guo LD, Liu RJ (2004) Arbuscular mycorrhizal fungi associated with common pteridophytes in Dujiangyan, southwest China. Mycorrhiza 14:25–30

Zhao ZW (2000) The arbuscular mycorrhizas of pteridophytes in Yunnan, southwest China: evolutionary interpretations. Mycorrhiza 10:145–149